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AN EXPERIMENT OF ARTIFICIAL SYNTOPY *EX SITU* BETWEEN *SPELEOMANTES ITALICUS* (DUNN, 1923) AND *S. AMBROSII AMBROSII* (LANZA, 1955)

(AMPHIBIA, PLETHODONTIDAE)

INTRODUCTION

The levels of genetic divergence observed within and between *Speleomantes* taxa are not correlated to their morphological differentiation, a feature typical of plethodontid salamanders (Wake *et alii* 1983). To assess correctly the taxonomic status and the species boundaries it is therefore essential to evaluate the degree of reproductive isolation between population groups (Lanza *et alii* 1995; Nascetti *et alii* 1996). When taxa are allopatric or parapatric without syntopy, differentiation indices such as genetic distances could be inadequate. In these cases to bring in syntopy allopatric populations whose individuals are distinguishable using genetic markers (e.g. discriminant alleles) may give relevant information (Coluzzi & Bullini 1971). However, in the case of plethodontid salamanders the experimental plan is so demanding and time consuming that has been judged to be unfeasible and scarcely useful (Wake 1981). Recent results have on the contrary demonstrated that such experiments can supply relevant data about the ecological and evolution-

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ary relationships among plethodontid species, provided they are run for long time enough (CIMMARUTA *et alii* 1999).

The range of *S. ambrosii* (Lanza, 1955), in northern Tuscany and eastern Liguria, is neighbouring to the ranges of *S. italicus* (Dunn, 1923) and *S. strinatii* (Aellen, 1958). Two different subspecies have been recently described, geographically separated by the Magra River. *Speleomantes ambrosii ambrosii* lives in the area of La Spezia province situated on the hydrographic right side of the Magra River; *S. ambrosii bianchii* (Lanza *et alii* 2005) inhabits the southern part of the province of Massa Carrara, i.e. on the left side of the Magra River (LANZA *et alii* 2005).

Speleomantes italicus and *S. a. bianchii* are known to meet and hybridize on the Apuan Alps, originating a strongly asymmetric introgression at the level of nuclear genes, without a complete blending of the two gene pools that remain substantially separated at the level of mtDNA (CIMMARUTA *et alii* 1989; RUGGI *et alii* 2005). *Speleomantes a. ambrosii* and *S. strinatii* are parapatric in eastern Liguria and neither syntopy nor hybridization have been observed in this area (CIMMARUTA *et alii* 1999). Within *S. a. ambrosii*, however, the populations from the lower Magra Valley are genetically quite differentiated, because they show alleles shared by both *S. italicus* and *S. strinatii* but not typical of *S. ambrosii* (CIMMARUTA 1993; FORTI 1998). The presence of these alleles within *S. a. ambrosii* gene pool can be explained through hybridization between *S. a. ambrosii* and either *S. italicus* or *S. strinatii*.

Since careful field surveys did not allow the finding of any contact zone in the high Magra Valley area, nor of *Speleomantes* populations in the area between Pontremoli and the lower Magra Valley, two experiments of artificial syntopy in the wild have been carried out, bringing *S. a. ambrosii* together with *S. strinatii* (CIMMARUTA *et alii* 1999) and with *S. italicus*. This last experiment, planned and started before the discovery of the hybrid zone in the Apuan Alps, has been carried out in a natural cave outside the range of both the involved species, to test for reproductive isolation mechanisms between *S. a. ambrosii* and *S. italicus* and to study the viability and fitness of their possible hybrids.

MATERIALS AND METHODS

The site chosen to carry out the experiment of artificial syntopy in the field was the limestone cave “Buca della Nebbia” just South of Fungaia (Siena), municipality of Monteriggioni, on the southern slope of Maggio Mt. at 315 m of height (cadastral number 257 T/SI, coordinates 43°20’09”N – 11°14’14”E of Greenwich). The site environment is relatively xeric and is characterized by a thermophylous vegetation: deciduous broad-leaved wood dominated by holm oaks (*Quercus ilex* L.) and downy oaks (*Q. pubescens* Willd). This site was outside the range of both the species involved, in order to keep the experiment under control and to avoid spreading a foreign species into the range of another one.

The artificial syntopy has been realised using specimens from two populations of *S. italicus* and *S. a. ambrosii* that were distinguishable by their dorsal colour pattern (Lanza *et alii* 1995). Thirty adults of *S. a. ambrosii* (16 males and 14 females) and 30 specimens of *S. italicus* (13 males, 14 females, 3 subadults) were introduced in the cave “Buca della Nebbia”. *S. a. ambrosii* specimens were taken from a very narrow, temporarily waterless stream bed, covered by leaf litter and stones, near the cave “Grotta della Taglia” (cadastral number 79Li/SP, 44°06’03”N - 09°47’27”E of Greenwich), 200-220 m of height, locality Colle Mantellina, “frazione” Pegazzano, municipality of La Spezia. This is a relatively xeric environment, covered by an oak wood *(Quercus ilex* L.) with chestnut trees (*Castanea sativa* Miller) and *Q. pubescens* Willd. The specimens of *S. italicus* were collected in the northern slope of the sandstone (arenaria macigno) hill “Colle di San Francesco”, close to Pian di Mugnone (about 43°48’N - 11°17’E), municipality of Fiesole (Florence), at 170-190 m of height. This is a relatively mesic area, covered by a close chestnut wood (*Castanea sativa* Miller) mixed with *Robinia pseudoacacia* L.

The experiment started on 2 April 1983. Animals were released in the deeper part of the cave, on top of the last room (fig. 1). During the subsequent 16 years, 35 surveys were carried out in the site, from 9 April 1983 to 11 April 1999. Till 16 May 1996 the temperature, the humidity and the number of specimens of both species, assessed on morphological basis, were recorded. During the control session of 16 May 1996 newborn individuals were recorded in the cave for the first time (by B. Campolmi and B. Lanza), 13 years after the beginning of the experiment (Lanza 1999, p. 102). Since this moment the control protocol was modified, including measurements of the observed salamanders: weight (W), total length (L), snout-vent length (SVL).

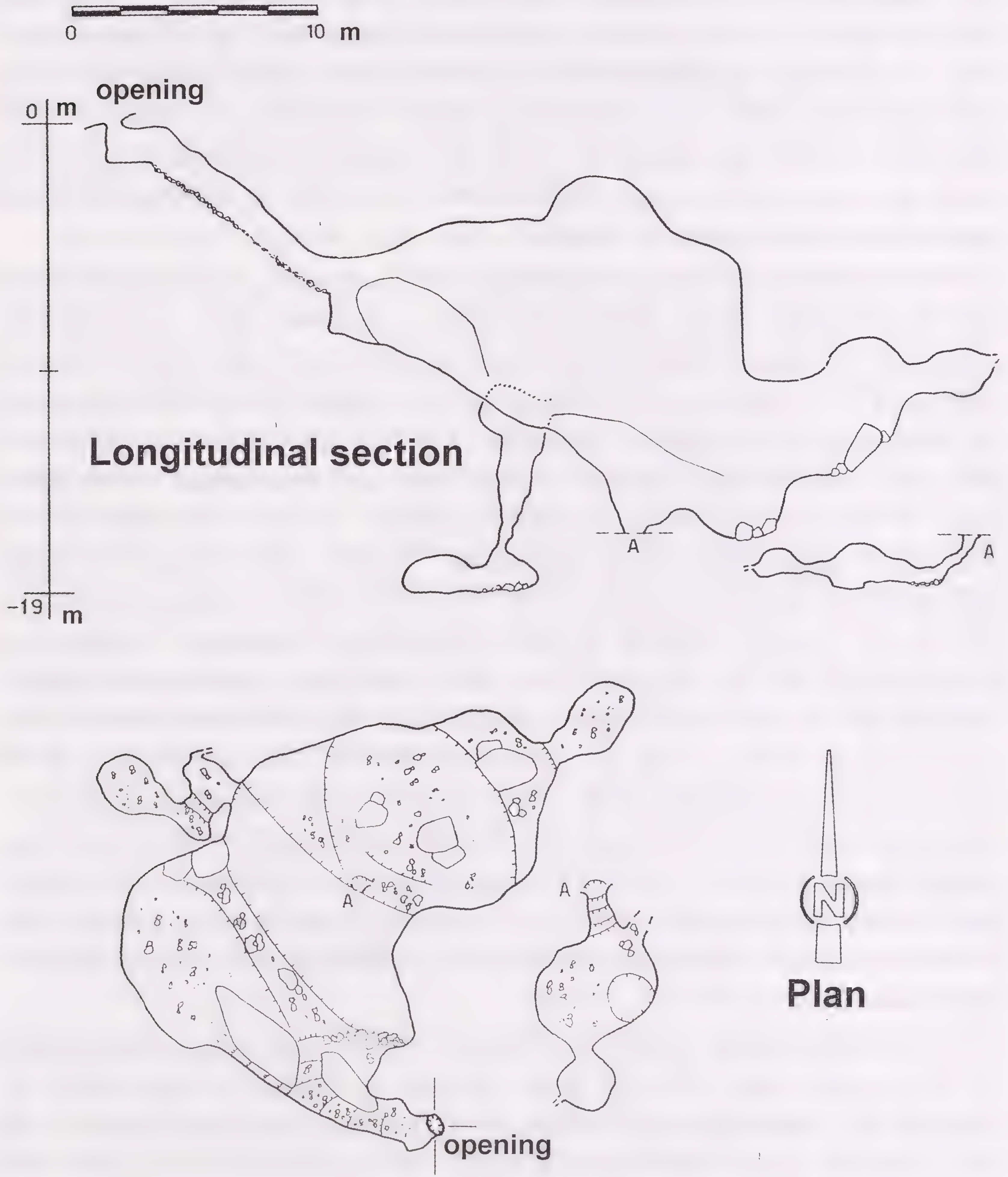

Fig. 1 - Longitudinal section and plan of the cave "Buca della Nebbia" (cadastral number 257 T/SI) used for the artificial syntopy experiment. Survey by F. Fallani and G. Adiodati (Gruppo Speleologico Fiorentino, C.A.I.), drawing by F. Fallani.

Specimens were also marked singularly by individual toe-clipping. This allowed estimating the population size (*N*) by mark–recapture method, using CHAPMAN (1951) index. Being the phalanx cut a temporary mark, specimens have been also photographed to allow their further identification using dorsal colour pattern. During each survey conducted after 16 May 1996, a small piece of the tail was taken from each newly captured specimen to assess its genotype (*S. a. ambrosii*, *S. italicus*, F_1 hybrid or backcross) using allozyme electrophoresis. To this aim, were scored five loci of the six discriminating between *S. a. ambrosii* and *S. italicus*: *Ldh-1, Ldh-2, Sod-1, Ck* and *Ada-1* (LANZA *et alii* 1995; FORTI *et alii* 1998) on an overall number of 55 salamanders. The electrophoretic methods were those reported in detail by LANZA *et alii* (1995, 2005).

RESULTS

Thirty-five control surveys were carried out in the experimental cave between 9 April 1983 and 11 April 1999. In 24 of these control sessions (68.8%) *Speleomantes* specimens were observed in the site. All the specimens were initially released at the end of the cave but were always recovered within the first 10 m. The higher number of observations was recorded in autumn and spring (seasonal mean; fig. 2). This is probably due to the scarce effectiveness of the cave itself as a refuge, since it undergoes marked changes in temperature and humidity especially during the summer, becoming a scarcely suitable habitat and preventing high concentration of *Speleomantes* inside.

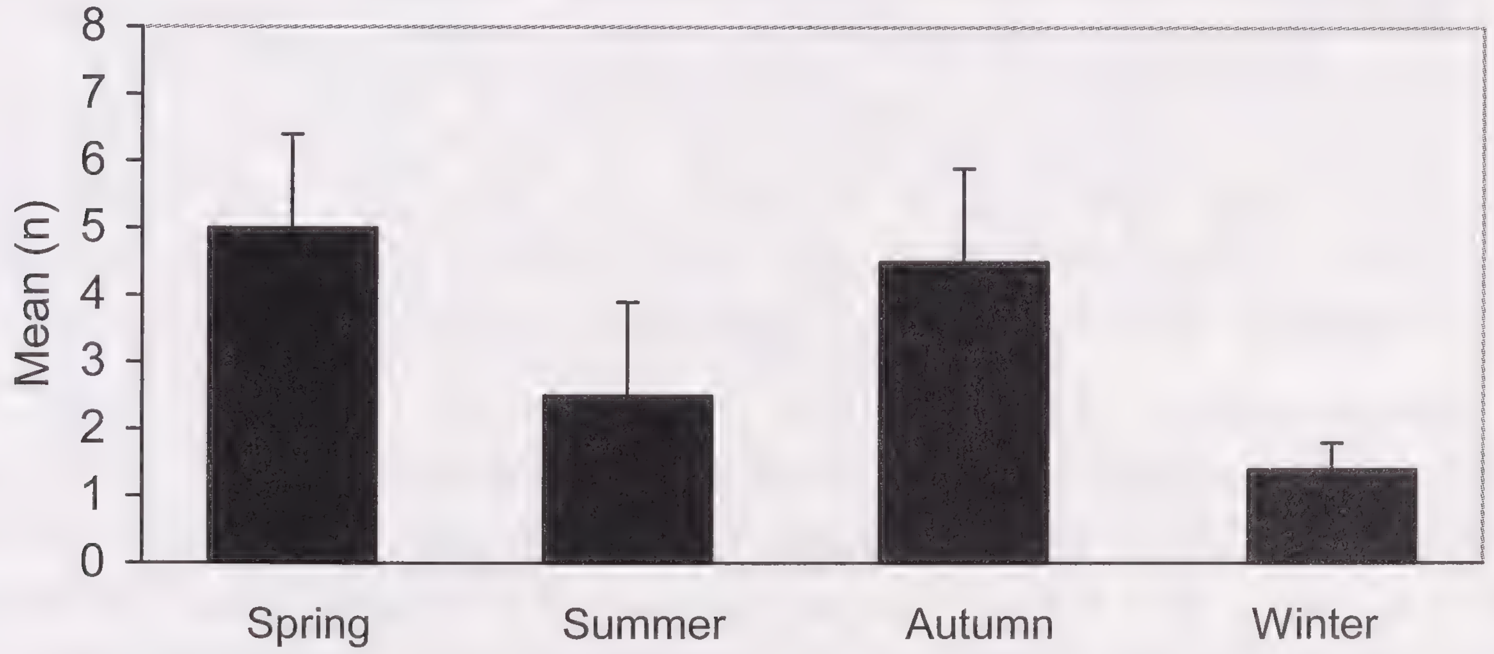

Fig. 2 - Seasonal means of *Speleomantes* specimens in the cave "Buca della Nebbia". Total number of observations: $N = 114$.

During a first period (9 April 1983 - 16 May 1996) 17 surveys have been carried out and in 59% (10 surveys) it was possible to find introduced individuals of both species within the cave, providing an overall number of 35 adult observations. Several surveys (41%; i.e., 7 surveys, with 28 observations) were carried out during the first year after the starting date, to check for the survival of the introduced specimens in the site, a result not to be taken for granted, because the site is outside the range of the genus.

The mean number of specimens recorded per session was very low (4 ± 1.05 vs. 60 specimens introduced), but both species were still living in the site in 1989, when the surveys were stopped for 4 years. Adults of both species were still there in 1993, when controls started again. During the survey carried out on 16 May 1996, newborn specimens were found for the first time. Since this moment a second phase started up, with a different sampling protocol (see Materials and Methods). Between 16 May 1996 and 11 April 1999, 18 surveys have been executed, with 79 specimens of the two species observed, with a mean of 4,39 (±0.89) individuals per survey.

The population size estimated in 1996 was N = 41 (±9.8) and overall 55 different individuals were observed between 1996 and 1999, including unmarked newborns. Young specimens were slightly more numerous than adults (55% vs. 45%). Twenty specimens were then recaptured during further controls, usually within 2-4 months after marking. In 3 cases, however, specimens were recaptured 20-29 months after being marked.

All the 55 observed specimens were classified as *S. a. ambrosii*, *S. italicus*, F_1 hybrids or backcrosses using allozyme markers. Most of the salamanders were *S. a. ambrosii*, with 38 (69%) young and adult specimens recorded (Fig. 3), while only two adults (4%) of *S. italicus* were observed. Fifteen specimens (27%) were hybrids, both young and adult. The bulk was F_1 hybrids (13) but also two adult backcrosses F_1 x *S. italicus* were found. None of the young born in the site was *S. italicus*: 70% were *S. a. ambrosii* and the others were hybrids.

Morphometric data obtained during years 1996-1999 have provided some preliminary indication on the growth rate of *Speleomantes*. A male of *S. a. ambrosii* captured on 16 May 1996 (L= 110 mm, SVL= 62 mm, W= 4.4 g) was recaptured 8.5 months later, showing a growth of 2 mm of SVL and an increase of 0.7 g in weight. A young *S. a. ambrosii* captured on 16 May 1996 (L= 45 mm, SVL= 25.5 mm, W= 0.4 g) during the same 8.5 months increased 1.5 mm in SVL and 0.2 g in weight. A female F_1 hybrid captured on 23 November 1996 (L= 119 mm, SVL= 66 mm, W= 5.6 g) was recaptured 2 years later, showing a growth of 0,5 mm of SVL.

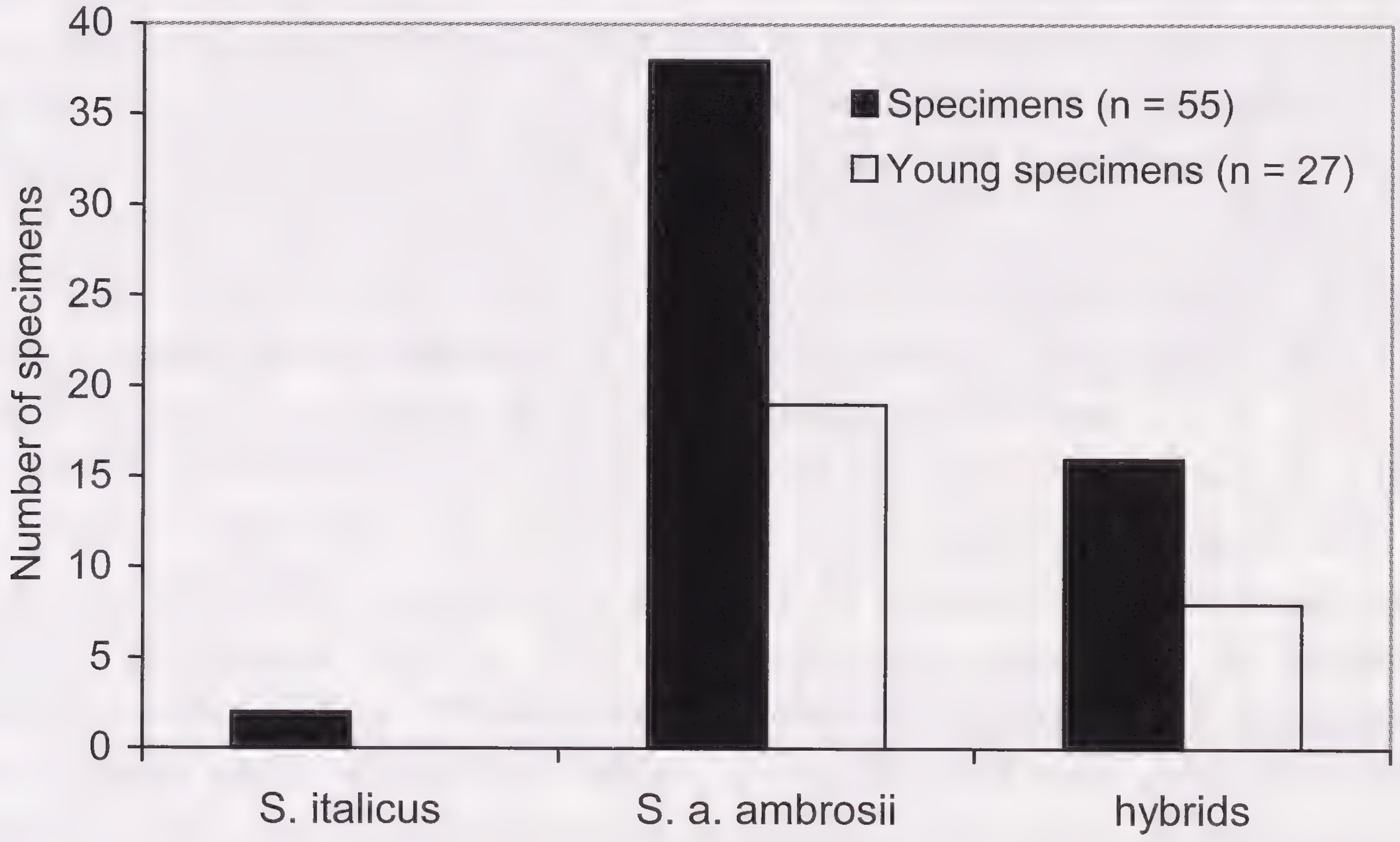

Fig. 3 - Specimens of *S. ambrosii*, *S. italicus* and hybrids observed in the second phase of the experiment (i.e. since newborns have been detected) (*N* = 55).

DISCUSSION

The results obtained show that the site chosen to carry out the experiment of artificial syntopy between *S. a. ambrosii* and *S. italicus* was suitable to host a viable *Speleomantes* population. The environmental conditions of the surface area are quite xeric and strongly influence the initial part of the cave environment, that becomes drier and warmer at summer time, as indicated by the bimodal distribution of *Speleomantes* frequencies shown in fig. 2 (see also PASTORELLI *et alii* 2001; FORTI *et alii* 2005).

Being the cave a poor refuge, it can not host a large *Speleomantes* population, as confirmed by the census data, providing an estimate of $N = 41$ (±9.8).

Speleomantes a. ambrosii seems to be better settled in the site, as suggested by its higher demographic abundance (fig. 3) and reproductive success; *S. italicus* is still inhabiting the site but shows lower densities with respect to *S. ambrosii* and does not produce new-borns in spite the adults are reproductively active, as shown by the birth of hybrids and backcrosses. This could be due to the fact that *S. a. ambrosii* individuals were from a site whose ecological features were relatively xeric, suggesting a slight pre–adaptation of the former to the environmental conditions of the xeric experimental site. Moreover, *S. a. ambrosii* has been already demonstrated able to survive successfully in xeric Mediterranean habitats, probably because of its high ecological plasticity (CIMMARUTA *et alii* 1999; FORTI *et alii* 2005).

The finding of young and adult F_1 hybrids shows that *S. italicus* and *S. a. ambrosii* hybridize when in syntopy. During the 16 years of the experiment the hybridization occurred several times and is still frequently happening, as witnessed by the high number of hybrids observed. Indeed, F_1 hybrids represented 12% of the overall number of specimens recorded and 25% of the young individuals. Moreover, the finding of two backcrosses F_1 x *S. italicus* already adult indicates that hybrids were viable and fertile. This finding also attests that at least three generations occurred in the experimental site during the 16 years of the experiment.

CONCLUSIONS

This experiment demonstrates that transplantation experiments can be successfully carried out in plethodontid salamanders in spite their long generation time.

The results obtained showed that *S. a. ambrosii* and *S. italicus* hybridize producing viable and fertile hybrids, able to backcross with parental specimens. This allows to make a hypothesis to explain the origin of the “foreign” alleles carried by the populations of *S. a. ambrosii* from La Spezia, shared by both *S. italicus* and *S. strinatii* but not typical of *S. ambrosii* (CIMMARUTA 1993; FORTI 1998). These alleles could be the signs of a past hybridization (paleointrogression) with *S. italicus*, rather than with *S. strinatii*: this study shows indeed that *S. a. ambrosii* hybridize at a high rate with *S. italicus*. On the contrary, it has been previously demonstrated that *S. a. ambrosii* and *S. strinatii* hybridize in a very limited extent when in syntopy (CIMMARUTA *et alii* 1999), making a past hybridization *S. a. ambrosii* x *S.*

strinatii less reliable. Further studies on mtDNA are now running to test this hypothesis.

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REFERENCES

CHAPMAN D.G., 1951 - Some properties of the hypergeometric distribution with applications to zoological census - *Univ. Calif. Publ. Statistics*, Berkeley, 1: 131-160.

CIMMARUTA R., 1993 - Ricerche di ecologia genetica ed evolutiva in Pletodontidi del genere *Speleomantes* (Amphibia: Plethodontidae) - Tesi di Dottorato di Ricerca, V Ciclo, Università degli Studi di Parma, Anno Accad. 1992-93, 130 pp.

CIMMARUTA R., FORTI G., NASCETTI G. & BULLINI L., 1999 - Spatial distribution and competition in two parapatric sibling species of European plethodontid salamanders - *Ethol., Ecol. & Evol.*, Firenze, 11: 383-398.

CIMMARUTA R., NASCETTI G. & BULLINI L., 1989 - A hybrid zone between the plethodontid salamanders *Hydromantes italicus* and *H. ambrosii* - Proc. II Congr. Europ. Soc. Evol. Biol. (E.S.E.B.), Roma: 17.

COLUZZI M. & BULLINI L., 1971 - Enzyme variants as markers in the study of precopulatory isolating mechanisms - *Nature*, London, 231: 455-456.

FORTI G., 1998 - Parapatria, competizione ed isolamento riproduttivo in specie gemelle del genere *Hydromantes* (Amphibia, Plethodontidae) - Tesi di Dottorato di Ricerca, XI Ciclo, Università degli Studi di Parma, Anno Accad. 1997-98, 62 pp.

FORTI G., CIMMARUTA R. & NASCETTI G., 2005 - Behavioural responses to seasonal variations of autoecological parameters in populations of *Speleomantes strinatii* (Aellen, 1958) and *S. ambrosii* (Lanza, 1955) (Amphibia, Plethodontidae) - *Annali Mus. civ. St. nat. G. Doria*, Genova 97: 179-192.

FORTI G., CIMMARUTA R., NASCETTI G., LANZA B. & BULLINI L., 1998 - Glaciazioni del Quaternario e microevoluzione delle popolazioni continentali del genere *Hydromantes* (Amphibia, Plethodontidae) - *Biogeographia*, Siena, 19 (1997): 197-211.

LANZA B., 1999 - *Speleomantes ambrosii* (Lanza, 1955) - Ambrosis Höhlensalamander (pp. 91-135) - In: GROSSEMBACHER K. & THIESMEIER B. (Eds.), Handbuch der Reptilien und Amphibien Europas. Band 4/1: Schwanzlurche (Urodela) - I. Aula Verlag, Wiesbaden, 408 pp.

Lanza B., Caputo V., Nascetti G. & Bullini L., 1995 - Morphologic and genetic studies of the European plethodontid salamanders: taxonomic inferences (genus *Hydromantes*) – *Monogr. Mus. reg. Sci. nat.*, Torino, 16, 368 pp.

Lanza B., Cimmaruta R., Forti G., Bullini L. & Nascetti G., 2005 - Bianchi's cave salamander, *Speleomantes ambrosii bianchii* n. ssp. (Amphibia, Caudata, Plethodontidae) - *Annali Mus. civ. St. nat. G. Doria*, Genova, 97: 59-77.

Nascetti G., Cimmaruta R., Lanza B. & Bullini L., 1996 - Molecular taxonomy of European plethodontid salamanders (Genus *Hydromantes*) - *J. Herpet.*, Lawrence, 30 (2): 161-183.

Pastorelli C., Laghi P. & Scaravelli D., 2001 - Studi preliminari sull'ecologia di *Speleomantes italicus* (Dunn, 1923) nell'Appennino tosco-romagnolo (Caudata: Plethodontidae) - *Pianura,* Cremona, 13: 347-351.

Ruggi A., Cimmaruta R., Forti G. & Nascetti G., 2005 - Preliminary study of a hybrid zone between *Speleomantes italicus* (Dunn, 1923) and *S. ambrosii* (Lanza, 1955) on the Apuan Alps, using RFLP analysis (Amphibia, Plethodontidae) - *Annali Mus. civ. St. nat. G. Doria,* Genova, 97: 135-144.

Wake D.B., 1981 - The application of allozymes to problems in the evolution of morphology - Evolution today. Proc. II Intern. Congr. System. and Evol. Biol.: 257-270.

Wake D.B., Roth G. & Wake M.H., 1983 - On the problem of stasis in organismal evolution - *J. theor. Biol.*, Sidcup, 101: 211-224.

ABSTRACT

An experiment of artificial syntopy has been carried out in the field, to assess the degree of reproductive isolation between *S. a. ambrosii* and *S. italicus*, two taxa parapatrically distributed and never found syntopic. The site used for the experiment has been surveyed for 16 years and the results obtained have shown that the two taxa hybridize extensively when in syntopy and that hybrids are viable and fertile, as demonstrated by the finding of two backcrosses already grown adult.

Previous allozyme studies highlighted that *S. a. ambrosii* populations from La Spezia area have in their gene pool alleles that are not typical of this taxon, but belonging to both *S. italicus* and *S. strinatii*. Our results suggest that these alleles could be due to a past hybridization (paleointrogression) of *S. a. ambrosii* with *S. italicus* rather than with *S. strinatii*.

RIASSUNTO

Un esperimento di sintopia artificiale in natura tra *Speleomantes italicus* e *S. ambrosii ambrosii* (Amphibia, Plethodontidae).

E' stato condotto un esperimento di sintopia artificiale in natura per verificare il grado di isolamento riproduttivo tra *S. italicus* e *S. a. ambrosii* nonché la vitalità e la fertilità di eventuali ibridi. La realizzazione dell'esperimento è stata resa necessaria dalla distribuzione parapatrica senza sintopia di questi due taxa. I 16 anni di rilievi effettuati hanno dimostrato che i due taxa ibridano in sintopia e che gli ibridi prodotti sono vitali e fertili, come provato dal ritrovamento di reincroci già adulti. Precedenti studi hanno mostrato che le popolazioni di *S. a. ambrosii* dell'area de La Spezia analizzate mediante elettroforesi multilocus presentano degli alleli estranei alla specie e tipici sia di *S. italicus* sia di *S. strinatii*. Perciò la verifica della possibilità di ibridazione tra *S. a. ambrosii* e *S. italicus* porta ad ipotizzare l'origine di questi alleli come paleointrogressione da *S. italicus* piuttosto che da *S. strinatii*.